Specification Amendments:

At the indicated page and line numbers, please replace the existing sections or paragraphs, as the case may be, with the ones set forth below.

(Page 11, lines 16-21)

Figure 1. Biosensor for c-Abl phosphorylation of the Crk-II adapter protein. c-Abl phosphorylates Crk-II on Tyr 221 which is thought to induce an intramolecular association with the SH2 domain. This rearrangement is expected to yield a net change in the distance between the termini of the protein, which would be reported by a dual-labeled derivative of Crk-II in which the FRET pair tetramethylrhodamine (Rh) and fluorescein (Fi) are specifically incorporated at its N- and C-termini, respectively.

(Page 22, line 10 through page 23, line 6)

EPL has been extended to permit the insertion of a synthetic peptide into a recombinant protein through the sequential ligation *in solution* of two recombinant protein fragments to the N- and C-termini of a synthetic peptide cassette (17). While this strategy is, theoretically, extendible to the ligation of any combination of synthetic and/or recombinant fragments, the need to perform all of the steps in solution renders the approach technically demanding; after each ligation reaction it is necessary to isolate the desired product from the reaction mixture, a process which is time-consuming and, importantly, leads to substantial handling losses. In principle, these problems should be overcome by transferring the entire process to the solid-phase, in a manner analogous to solid-phase peptide synthesis (SPPS) (18). As with SPPS, this solid-phase protein ligation (SPPL) approach should allow each reaction to be driven to a complection by using a large excess of reagents, which can then be simply removed by washing. In addition, there would be no need to isolate intermediate ligation products which would remain immobilized on the support. The present inventors have developed an SPPL technology and have successfully applied it to the generation of a dual-labeled version of the ~35 kDa adapter protein, Crk-II. As is shown herein, this semi-synthetic protein analog specifically biosenses a post-translational tyrosine phosphorylation event important in regulation of Crk-II mediated signal transduction. Thus, it may be used for various purposes, such as to identify agents capable of modulating phosphorylation activity.

It is also only an example of other protein kinase targets, and more broadly, other useful polypeptides that biosensing conformational changes therein is useful in screening and other purposes, as noted below.

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As will be seen in the Examples below, the synthesis was carried out of a semi-synthetic version of the adapter protein, Crk-II, in which the FRET pair, tetramethylrhodamine and fluorescein were incorporated at the N- and C-termini of the protein, respectively (hereafter referred to as Rh-(Crk-II)-Fl), as described in summary above. Crk-II has been implicated in a number of cellular signaling processes, and is composed predominantly of one Src homology 2 (SH2) and two SH3 domains through which it mediates intermolecular protein-protein interactions (22, 23). Two protein tyrosine kinases, c-Abl and the epidermal growth factor receptor (EGFR), are known to phosphorylate Crk-II on a unique tyrosine residue (Tyr221) located between the SH3 domains (24, 25). This post-translational modification is thought to regulate Crk-II function by inducing an intramolecular association with the SH2 domain (26) which in turn inhibits certain intermolecular protein interactions (22-25). It was anticipated that phosphorylation and subsequent intramolecular association would result in a distance change between the termini of Crk-II, which would lead to a change in FRET between the two fluorophores in the dual-labeled analog (Fig. 1). Consequently, this protein construct would directly biosense this important post-translational event.

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As will be shown in further Example, below, phosphorylation studies were performed on the construct to demonstrate its utility in identifying modulators of protein kinase activity. Purified Rh-Crk-II)-Fl was assays for its ability to biosense Crk-II phosphorylation by the c-Abl protein tyrosine kinase. As indicated previously, phosphorylation by c-Abl leads to an intramolecular association between a phosphotyrosine motif and the Crk-II SH2 domain, which can be reported by the dual-labeled Crk-II derivative (Fig. 1). Rh-(Crk-II)-Fl was treated with full length recombinant c-Abl and aliquots of the reaction mixture were analyzed by fluorescence spectroscopy and western blotting at ~1 min and 60 min time-points. In the absence of ATP, essentially no change in FRET (i.e. the ratio of the fluorescein/tetramethylrhodamine emission intensities) was observed during the reaction (Fig.

3A), and no Rh-(Crk-II)-Fl phosphorylation could be detected using an anti-phosphotyrosine monoclonal antibody (Fig. 3B). In contrast, when ATP was included in the reaction mixture, a phosphorylation-dependent increase in the emission intensity ratio (a decrease in FRET) was cnosistently observed. Rh-(Crk-II)-Fl was completely phosphorylated after 1 h as determined by native PAGE mobility (Fig. 3C). The quite modest decrease in FRET (~3% after 60 min) suggests that the SH2-phosphotyrosine interaction, which is triggered by Rh-(Crk-II)-Fl phosphorylation, results in only a small net change in the relative distance between the N- and C-termini in the protein.

(Page 35, line 20 through page 36, line 4)

SPPL has allowed the synthesis of a semi-synthetic Crk-II analog in which the FRET pair, Rh and Fl, were specifically introduced at the N- and C-termini of the protein. The two fluorophores were positioned close to the natural ends of Crk-II (≤ 10 Å) in order to maximize the sensitivity to conformational change in this region. This type of chemical-labeling is analogous to the incorporation of different GFP derivatives at the termini of recombinant proteins through standard DNA cloning methodologies (31, 32, 33).

(Page 36, line 18 through page 37, line 4)

The resonance energy transfer between the fluorophores in the unphosphorylated molecule was calculated to be 52.5% as determined from both the quenching of the fluorescein emission intensity and the sensitized emission of the rhodamine acceptor (as in ref. 34). Assuming that both fluorophores have random orientations and using a Förster distance of 45 Å for the Fl-Rh pair (34), then the distance between the two fluorophores is ~44 Å. Interestingly, this suggests that unphosphorylated Crk-II has a somewhat compacted domain architecture, as opposed to a linear array of domains; based on the primary sequence, the N- and C-termini could be as much as ~200 Å apart if the inter-domain linkers assume a fully extended conformation.

(Page 46, line 18 through page 47, line 19)

Fluorescence Spectroscopy. Experiments were conducted at 18°C in a stirred 0.5 cm-pathlength cell using a SPEX FL3-11C fluorimeter. Samples from the reactions (50 μl) were diluted into 2 mM DTT, 0.4 mg/mL BSA, 140 mM NaCl, 50 mM Tris, pH 7.4 buffer (450 μl) for analysis. Excitation was at 490 nm with a 2.5 nm slit and the fluorescence

emission was monitored at 520 nm and 580 nm through a 4 nm slit. Purified Rh-(Crk-II)-Fl was assayed for its ability to biosense Crk-II phosphorylation by the c-Abl protein tyrosine kinase. As indicated previously, phosphorylation by c-Abl leads to an intramolecular association between a phosphotyrosine motif and the Crk-II SH2 domain, which could potentially be reported by the dual-labeled Crk-II derivative (Fig. 1). Rh-(Crk-II)-Fl was treated with full length recombinant c-Abl and aliquots of the reaction mixture were analyzed by fluorescence spectroscopy and western blotting at ~1 min and 60 min time-points. In the absence of ATP, essentially no change in FRET (i.e. the ratio of the fluorescein/tetramethylrhodamine emission intensities) was observed during the reaction (Fig. 3A), and no Rh-(Crk-II)-Fl phosphorylation could be detected using an anti-phosphotyrosine monoclonal antibody (Fig. 3B). In contrast, when ATP was included in the reaction mixture, a phosphorylation-dependent increase in the emission intensity ratio (a decrease in FRET) was consistently observed. Rh-(Crk-II)-Fl was completely phosphorylated after 1 h as determined by native PAGE mobility (Fig. 3C). The quite modest decrease in FRET (~3% after 60 min) suggests that the SH2-phosphotyrosine interaction, which is triggered by Rh-(Crk-II)-Fl phosphorylation, results in only a small net change in the relative distance between the <u>N</u>- and C-termini in the protein.